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SEROLOGIC EVIDENCE OF JAMESTOWN CANYON AND KEYSTONE VIRUS INFECTION IN VERTEBRATES OF THE DELMARVA PENINSULA*

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Abstract. Serological data accumulated during the past decade indicated that a variety of feral and domestic animals of the Delaware-Maryland-Virginia (DelMarVa) Peninsula were infected with Jamestown Canyon (JC) and/or Keystone (KEY) viruses (Bunyaviridae, California serogroup). Neutralizing (N) antibody to JC virus was most prevalent in white-tailed deer, sika deer, cottontail rabbits and horses. KEY virus N antibody was detected most frequently in gray squirrels and domestic goats. N antibody indicative of past infection by one or both viruses also was found in raccoons, horses and humans. JC and/or KEY virus N antibodies were not demonstrable in sera of several other species of small mammals and reptiles. Investigations were extended to evaluate the role of domestic goats as an amplifying host of JC and KEY viruses and to assess their potential as sentinels of virus transmission. Goats maintained in the Pocomoke Cypress Swamp during the summer season of 1978, acquired N antibodies to JC and KEY viruses. Following experimental inoculation with either JC or KEY virus, all goats developed N antibody despite the absence of a demonstrable viremia in most animals. Goats proved to be effective as sentinels for monitoring the transmission of JC and KEY viruses; however, the exceptionally low titers or absence of viremia following inoculation with these viruses would seem to preclude a potential virus-amplifying role for this species. Although findings implicated primarily gray squirrels and white-tailed deer as possible amplifying hosts of KEY and JC virus, respectively, further investigations will be required to clarify their role, particularly since both viruses may be maintained entirely by transovarial transmission.

Keystone (KEY) and Jamestown Canyon (JC) viruses (Bunyaviridae, California serogroup) occur in scattered enzootic foci on the Delaware-Maryland-Virginia (DelMarVa) Peninsula.[1–5] Transovarial transmission of KEY virus has been demonstrated in *Aedes atlanticus*,[2] and field-collected *Aedes canadensis* have yielded isolates of JC virus.[1] Studies conducted on the ecology of these viruses on the DelMarVa Peninsula have implicated a variety of small mammal(s) as potential amplifying hosts of KEY virus, while serosurveys suggest white-tailed deer to be the predominant host for JC virus.[3–5] The relative involvement of other feral animals, as well as the possible role of domestic animals, has not been resolved. Transmission of these viruses to vertebrates and the potential for subsequent virus amplification become important parameters in understanding the ecology and epidemiology of viruses that are presumably transovarially maintained.[6] This study addresses the latter parameters in regard to virus ecology, and assesses the practical use of domestic goats as sentinels to detect transmission of KEY and JC viruses.

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MATERIALS AND METHODS

Serum collections

Human serum specimens were obtained from serum storage files maintained at the Walter Reed Army Institute of Research. Specimens tested were from persons previously associated with field investigations in the Pocomoke Cypress Swamp (PCS) of the DelMarVa Peninsula. Small vertebrates were captured at the PCS and at Assateague Island, Va. Detailed descriptions of these areas were reported previously.[7,8] Sera were obtained by cardiac puncture from small feral animals, and domestic animals were bled by jugular venipuncture. Except for white-tailed deer, all animals were live-trapped, age and sex was recorded, and they were released at the capture site. Sex and age determinations for gray squirrels were based on methods of Brown and Yeager.[9] All deer sera were obtained at hunter check stations within a 50-km radius of the PCS on the mainland and at Assateague Island during November and December. Age estimates of deer were based on the presence or absence of permanent incisors and canine teeth, as well as tooth eruptions and wear.[10]

Sentinel goat experiments

Locally purchased seronegative domestic goats were kept in Magoon traps at five separate locations in the PCS. All goats were bled at weekly intervals during the summer of 1978, and sera were tested for KEY and JC virus neutralizing (N) antibodies by plaque reduction neutralization (PRN) tests.

Experimental infection of goats with KEY or JC virus was done in Magoon traps during the months of December and January, when mosquitoes were not active in this area. As an added precaution, traps were screened and sealed to preclude the entrance of arthropods. Goats were bled by venipuncture on days 1–7 postinoculation and at various time intervals thereafter.

Blood specimens for both N antibody and viremia determinations were placed on wet ice for the approximately 2 hours required for transport to the laboratory prior to centrifugation and freezing at −70°C.

Virus assay and neutralization tests

Baby hamster kidney cells (BHK-21) clone 15[5] were used for all assays. Cells were propagated in Eagle's Basal Medium-Hanks' Salts (BME-H) supplemented with 10% heated fetal calf serum (FCS), and penicillin (200 units) and streptomycin (200 μg/ml). Virus isolations and titrations were performed by incubating mosquito suspensions, animal blood dilutions and confluent monolayers of BHK cells for 1 hour at 35°C. Cell monolayers were subsequently overlaid with 5 ml of Difco purified agar (1%) and Medium 199 supplemented with FCS (10%), DEAE dextran (0.005%), and vitamins and essential amino acids at one-half the concentration recommended by Grand island Biological Co., Grand Island, N. Y. Following incubation at 35°C for 3 days, a second agar overlay, containing an equal volume of Difco purified agar (1%) and medium 199 supplemented with FCS (2%) and neutral red (0.004%), was added. Plaques were counted within 24 hours after the second agar overlay.

Animal sera were screened at a 1:10 dilution for antibody by PRN tests. Each diluted serum was mixed with a virus dilution previously determined to yield 50–100 plaque-forming units (pfu). Serum-virus mixtures were incubated for 1 hour at room temperature and then inoculated onto BHK-21 cell cultures, 0.2 ml per culture. Subsequently, agar overlays were added to cell cultures and plaques were recorded as described above. A reduction of 50% or more of the virus pfu by 1:10 dilution of each serum was used as the criterion for virus N antibody. Samples of sera from selected vertebrate species which neutralized both the JC and KEY virus pfu by 50% or more were diluted further and tested to determine 50% plaque reduction neutralization titers (PRN_{50}). Titers were estimated from a graphic plot on probit paper and expressed as the reciprocal of the PRN_{50} serum dilution. N antibody was considered specific for a particular virus, if titers were fourfold higher than heterologous virus titers. This criterion was based on the immune response of white-tailed deer[5] and domestic goats following experimental KEY and JC virus infection.

Keystone and JC viruses were originally isolated from mosquitoes as previously described.[5] La Crosse (LAC) virus, not previously observed on the DelMarVa Peninsula, was obtained from Dr. Wayne Thompson, University of Wisconsin, Madison.[11] This virus had received five intracerebral passages in suckling mice.

Reference antisera were prepared to KEY, JC and LAC viruses by the methods of Brandt et al.[12] as modified by Chiewslip and McCown.[13]

RESULTS

A summary of the prevalence and distribution of KEY and JC virus N antibody among vertebrates and reptiles is presented in Table 1. Evidence based on screening sera at a 1:10 dilution

TABLE 1

Keystone (KEY) and Jamestown Canyon (JC) virus neutralizing (N) antibody prevalence rates for feral and domestic animals, DelMarVa Peninsula, 1973–1980

Species	Location	No. tested	No. with N antibody: KEY	JC	Both KEY/JC	% N antibody
White-tailed deer (*Odocoileus virginianus*)	Mainland	392	30	66	189	75
White-tailed deer	Island	11	0	6	0	55
Sika deer (*Cervus nippon*)	Island	47	0	16	5	45
Gray squirrel (*Sciurus carolinensis*)	Mainland	275	68	0	61	47
Cottontail rabbit (*Sylvilagus floridanus*)	Island	21	2	8	0	48
Raccoon (*Procyon lotor*)	Mainland	105	12	NT*	NT	11
Opossum (*Didelphis marsupialis*)	Mainland	32	0	NT	NT	0
Red fox (*Vulpes fulva*)	Mainland	2	0	NT	NT	0
Rice rat (*Oryzomys palustris*)	Island	5	0	NT	NT	0
Norway rat (*Rattus norvegicus*)	Island	11	0	NT	NT	0
Deer Mouse (*Peromyscus maniculatus*)	Mainland	110	0	NT	NT	0
Meadow mouse (*Zapus hudsonius*)	Mainland	15	0	NT	NT	0
Reptiles†	Mainland	227	0	0	0	0
Domestic goats	Mainland	16	6	0	2	50
Horses	Island	16	0	3	3	38
Humans	Mainland	25	2	3	1	24

* Not tested.
† Included 101 painted turtles (*Chrysemys picta*), 42 snapping turtles (*Chelydra serpentina*), 34 mud turtles (*Kinosternon subrubrum*), 25 box turtles (*Terrapene carolina*), 3 spotted turtles (*Clemmys guttala*), 16 unidentified snakes.

revealed that JC virus N antibody was detected most frequently in sika deer, white-tailed deer, cottontail rabbits and horses. KEY virus N antibody was most prevalent in gray squirrels and domestic goats. KEY virus N antibodies were detected in sera of raccoons, but not in sera of other species of small mammals and reptiles. Likewise, human sera were negative for LAC virus N antibody. Except for cottontail rabbits and white-tailed deer from Assateague Island, N antibody suggestive of a dual infection by both viruses was detected in all antibody-positive species tested against both viruses. PRN_{50} titers for a sample of these sera that neutralized both KEY and JC viruses are presented in Table 2. Results indicated that 62 of the white-tailed deer exhibited JC virus antibody, four KEY virus antibody, and six yielded comparable PRN_{50} titers to both viruses. PRN_{50} titers for three sika deer sera were indicative of JC infection. KEY virus antibody alone was detected in 10 gray squirrel sera, which was consistent with screening results presented in Table 1. Equivocal antibody titers for three horses, two goats and one human serum suggested past infection with both KEY and JC viruses.

Annual prevalence rates of KEY and JC virus N antibodies for white-tailed deer remained fairly constant from year to year (Table 3). In contrast,

TABLE 2

Specificity of viral neutralizing antibody to Jamestown Canyon (JC) and Keystone (KEY) viruses detected in vertebrate sera

Species	No. of sera	Antibody specificity	Geometric mean reciprocal PRN_{50} titer (range): JC	KEY
White-tailed deer	62	JC	199 (40–640)	32 (10–160)
	4	KEY	25 (15–40)	212 (160–320)
	6	Both	56 (40–80)	57 (30–80)
Sika deer	3	JC	211 (160–330)	18 (10–60)
Gray squirrels	10	KEY	37 (10–130)	308 (40–1,280)
Domestic goats	2	Both	89 (40–200)	113 (80–160)
Horses	3	Both	120 (90–160)	91 (40–160)
Humans	1	Both	230	450

TABLE 3

Keystone (KEY) and Jamestown Canyon (JC) virus neutralizing (N) antibody prevalence rates for white-tailed deer, DelMarVa Peninsula

Year	No. tested	No. with N antibody: KEY	JC	Both KEY/JC	% N antibody
1973	121	9	36	47	76
1974	61	0	7	33	66
1977	200	21	25	106	76
1978	11	0	4	3	64
Total	393				74

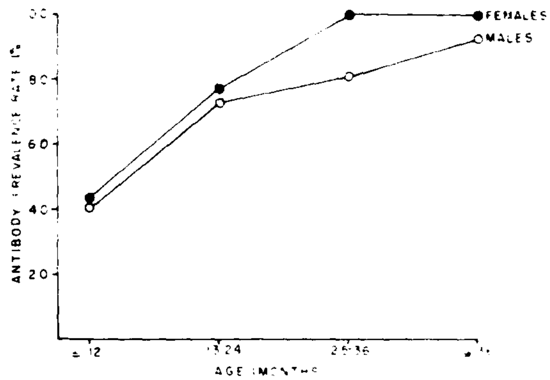

FIGURE 1. Jamestown Canyon and Keystone virus age-specific neutralizing (N) antibody prevalence rates for white-tailed deer, DelMarVa Peninsula, 1974, 1977 and 1978.

KEY virus antibody rates in gray squirrels declined from 66% (58/66) for 1975 to 49% (40/82), 42% (27/64) and 10% (4/41) for 1976, 1977 and 1978, respectively.

Overall sex-specific JC and KEY virus N antibody prevalence rates for white-tailed deer were 72% (90/125) for males and 68% (58/85) for females. In contrast, the N antibody age-specific rates varied from approximately 40% for deer 12 months of age or less to rates approaching 100% for deer 36 months old and older (Fig. 1). Age- and sex-specific N antibody rates for sika deer were similar to those noted for white-tailed deer. No apparent difference was noted in sex-specific N antibody prevalence rates for gray squirrels as indicated by a 48% (86/169) rate for males and a 47% (42/90) rate for females. The age-specific rates for squirrels was 25% (8/32) for juveniles and 51% (116/227) for adults.

Serological evidence of KEY and possibly JC virus infections in goats was suggestive of a virus amplifying role for these animals. In addition, the data implied that goats would serve effectively as indicators of virus transmission. Virus amplification and transmission were studied by determining the viremia and immune response of goats following intravenous inoculation with JC and KEY viruses. A viremia was not demonstrable in any of six goats inoculated with 1 ml of $9.3 \times 10^{4.0}$ pfu/ml of KEY virus nor in any of five goats inoculated with 1 ml of $1 \times 10^{2.0}$ pfu/ml of JC virus. Less than $1 \times 10^{1.0}$ pfu/ml, however, were detected in blood samples obtained from two of six goats inoculated with $2.9 \times 10^{4.0}$ pfu/ml of JC virus.

All goats developed either JC or KEY virus N antibody (Table 4). Maximum antibody titers were noted between days 10 and 20 postinoculation (P.I.) for most goats. Subsequently, titers declined, but most animals possessed N antibody through day 90. N antibody titers for goats inoculated with the high dose of JC virus were greater than those induced by the low dose. The difference was sig-

TABLE 4

Keystone (KEY) and Jamestown Canyon (JC) virus neutralizing (N) antibody response of experimentally infected goats

Inoculum	N antibody	Reciprocal PRN_{50} titer by day P.I.: 10	20	30	45	60	90
JC virus	JC	1,700*	800	500	300	300	200
(2.9×10^4 PFU/ml)	KEY	90	40	30	15	15	15
JC virus	JC	100	500	300	100	200	100
(1×10^2 PFU/ml)	KEY	15	100	40	30	20	15
KEY virus	KEY	100	200	90	70	60	40
(9.3×10^4 PFU/ml)	JC	<10	10	<10	<10	<10	<10

* Plaque reduction neutralization titer 50% endpoint based on mean titers of 4–6 individual goats.

nificant only for titers on day 10 P.I. ($P < 0.001$) compared to those for days 20 and 30 ($P > 0.1$).

During the summer of 1978, 6- to 8-month old goats were maintained in Magoon traps to monitor transmission of KEY and JC viruses in the PCS. Although mosquito abundance was extremely low, one goat acquired N antibody to JC virus during early July, while three other goats seroconverted to KEY virus during late August and early September. *Ae. canadensis* and *Culex salinarius* were the predominant mosquito species collected inside the Magoon traps in which the goats acquired JC virus N antibody. Attempts to isolate virus from 3,148 females of these two mosquito species collected during a 1-month period immediately before the goats seroconverted were unsuccessful. KEY virus seroconversions coincided closely with the emergence of a very low number of *Ae. atlanticus*; but, 93 females of this species captured during a 1-month period prior to the acquisition of N antibody by goats were negative for virus.

DISCUSSION

On the basis of the serological data, several species of feral and domestic animals of the DelMarVa Peninsula were previously infected by JC and/or KEY viruses. N antibody prevalence rates indicated that gray squirrels and domestic goats were infected most frequently with KEY virus, while JC virus infections were most common in white-tailed deer, sika deer, cottontail rabbits and horses. Although type-specific JC virus N antibody was not found in gray squirrels, serum from individual squirrels frequently neutralized both JC and KEY viruses. Apparently, this reflected an immune response to KEY virus antigen(s) common to JC virus, because a sample of these sera exhibited PRN_{50} titers consistently higher for KEY than JC virus, thus suggesting that gray squirrels were infected with only KEY virus. In contrast, type-specific KEY and JC virus N antibodies were demonstrated in sera of white-tailed deer of the DelMarVa Peninsula. Also, as observed for gray squirrels, sera of individual white-tailed deer neutralized both JC and KEY viruses. Evidence based on PRN_{50} titers indicated that the majority of these deer were previously infected with JC virus and that the remainder reflected infection by one or the other virus alone, and/or suggested dual infections. Evidence of both JC and KEY virus infections in white-tailed deer was not surprising as previous findings showed *Ae. atlanticus*, the primary vector of KEY virus, to feed more frequently on this animal than several other vertebrates of the DelMarVa mainland.[3] Experimental studies also have demonstrated that this animal developed antibody following inoculation with KEY virus.[5] Studies conducted in Wisconsin revealed that white-tailed deer were infected with JC and LAC viruses,[14] but the majority of deer were infected with only JC virus.

Evidence of JC virus infection alone was found in sika deer and white-tailed deer on Assateague Island. Unlike the wide geographical distribution of white-tailed deer, the distribution of sika deer on the DelMarVa Peninsula is restricted to Assateague Island. The failure to detect evidence of KEY virus infection in either white-tailed deer or sika deer on Assateague Island suggests that the vector of this virus on the island differs from that on the DelMarVa Peninsula mainland. Recently *Ae. atlanticus* was found to breed on Assateague Island (unpublished data); however, the population density appeared to be exceptionally low as indicated by 30 adults captured in light traps throughout the 1978 summer season. During the same period, serological evidence of KEY virus infection was detected in sentinel hamsters, and KEY virus was isolated from female *Aedes infirmatus* and not from several other mosquito species, including the 30 *Ae. atlanticus*. The KEY virus minimum field isolation ratio for *Ae. infirmatus* was 1:6,308, which was not considered indicative of an important vector role. In addition, gray squirrels, a potential amplifying host of KEY virus on the mainland, were not found on Assateague Island. Experimental findings,[5] and limited data from this study, suggested that cottontail rabbits occupied this role; however, further investigations are needed to clarify its importance in the natural transmission cycle of KEY virus.

Among the species of feral vertebrates harboring JC virus N antibody, only white-tailed deer were previously implicated as an important host of this virus.[14-16] Our data, and experimental demonstration of a JC virus viremia in white-tailed deer,[5] support the role of the species as a potential amplifying host of this virus. Sika deer, and cottontail rabbits from Assateague Island also experienced JC virus infections, but further studies will be required to assess the importance of these additional species in the natural cycle of JC virus. Small mammals have not previously been implicated in the transmission cycle of JC virus.

In the south and southeastern U.S., cotton rats and cottontail rabbits were implicated as the principal amplifying hosts of KEY.[17] Our findings indicated that gray squirrels were frequently infected with this virus on the mainland of the DelMarVa Peninsula. In addition, previous experimental data showed this species to develop a viremia of sufficient magnitude to infect *Ae. atlanticus*, the primary vector of KEY virus.[5] Experimental data showed that cottontail rabbits develop a viremia following inoculation with KEY virus,[5] but attempts to capture this species on the mainland during sero-survey studies have not been successful. Cotton rats do not occur in this region.[18]

Although white-tailed deer were infected with KEY virus, the low N antibody prevalence rate, and the failure of these deer to develop viremia after inoculation with KEY virus,[5] precludes a potential amplifying role for this species.

The extent of KEY virus infection in gray squirrels varied, as indicated by the annual N antibody prevalence rate of 66% for 1975 compared to a 10% rate for squirrels captured during 1978. The high N antibody prevalence rates noted during 1975 were closely associated with the exceptionally high population density of *Ae. atlanticus* (unpublished data). Subsequently, during 1976–1978, the population density of this species was very low, thus, suggesting that the decline in N antibody prevalence rates was due to a decrease in abundance of *Ae. atlanticus*.

Neutralization antibody prevalence rates for KEY virus were appreciably lower in both juvenile white-tailed deer and gray squirrels than in the adults of these species. An explanation for the lower KEY virus antibody rates in squirrels will require further studies. A similar observation was noted for JC virus antibody rates in white-tailed deer of Wisconsin and North Dakota.[14,15] Studies conducted in Wisconsin attributed the lower rates to maternal N antibody which were shown to render offspring refractory to JC virus infection during the first summer season after birth.[19]

Antibodies suggestive of KEY virus infections were detected in a low percentage of raccoon sera. These findings, and the absence of demonstrable N antibody in other small mammals and reptiles studied, suggested that they were not involved in the transmission cycle of this virus.

Jamestown Canyon and KEY virus N antibodies were detected in the sera of humans, but the low prevalence indicated that infection was uncommon. Similar observations were reported for KEY virus in Florida[20] and JC virus in Wisconsin.[21] LAC virus N antibodies were not detected in humans, coinciding with previous attempts to detect this virus in mosquitoes and vertebrates of the DelMarVa Peninsula.

Jamestown Canyon and KEY virus N antibodies were detected in goats, but viremia was demonstrable only at trace titers in two animals inoculated with JC virus. Similar results were reported for other domestic animals, including horses following experimental KEY and JC virus infection.[22] These findings implied that domestic animals were "dead-end" hosts for these viruses, and also demonstrated their utility for monitoring KEY and JC virus transmission.

Although most goats inoculated with KEY and JC virus did not have demonstrable viremia infection did occur, as evidenced by the appearance of virus-specific N antibody. This feature, and the rapid and sustained immune response to low doses of JC and KEY viruses, are desirable characteristics for using goats to monitor virus transmission, especially JC virus. Except for white-tailed deer,[23] other vertebrates have not been effective or considered feasible for use as indicators of transmission of JC virus. Goats were easily maintained and bled, and seroconversions to JC and KEY viruses during 1978 indicated that this animal would serve effectively in monitoring the transmission of these viruses.

Although evidence of JC and KEY virus infection was detected in several vertebrate species, our observations do not directly implicate any vertebrate(s) as amplifying host. Since KEY,[2] and most likely JC virus,[24] can be transovarially transmitted in nature, it is possible that virus maintenance relies entirely on this mechanism. Experimental studies with another California group virus in which filial infection rates approached 100%[25] support this hypothesis; a model generated for KEY virus transmission[6] inferred that an amplifying host or hosts[5] is required for virus maintenance. On the basis of the limited data, gray squirrels and white-tailed deer appear to be the most likely amplifying hosts of KEY and JC viruses, respectively, if, in fact, any vertebrate species is required.

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